A NEW SPECIES OF MICRO-ORGANISM (*PROTEUS MELANOVOGENES*) CAUSING BLACK ROT IN EGGS

By A. A. MILES
Department of Pathology, Cambridge

With an introduction by E. T. HALNAN
Department of Agriculture, Cambridge

(With 1 Figure in the Text)

INTRODUCTION

By E. T. Halnan

During October 1934 a black rot occurred in imported eggs within a fortnight of the consignment being received at the port of entry. Cases drawn from this shipment when inspected at the port passed as first quality. The eggs among which the outbreak occurred were approximately 7 weeks old at the time when the rot showed, and had been held during this time at 30–35° F., under cold-storage conditions. Five eggs containing black rot were brought back to Cambridge and candled on 19 October. All these eggs smelt strongly of H_2S and a solution of lead acetate painted on the outside of the shell rapidly turned black owing to the formation of lead sulphide. The appearance on candling was as follows:

Egg 1. Air cell firm, dimensions 29 × 5 mm. Contents dead black with yellow rim next to air cell.

Egg 2. Air cell firm, dimensions 28 × 5 mm. Contents dead black throughout.

Egg 3. Air cell firm, dimensions 29 × 7 mm. Contents dead black throughout. Opened. Contents grey-black, with yolk a dense black on outside, green to yellow in middle. Addition of H_2O_2 caused solution to change to orange-yellow. White liquefied.

Egg 4. Air cell firm, at side of egg, dimensions 26 × 3 mm. Upper half dead black, lower half orange-yellow. Opened. The white had liquefied and was straw yellow with whitish flakes of material dispersed through it. The yolk was intact, the outer border only being inky black. A few brownish patches were discernible on the inner shell membrane and on the shell itself. Examination of the liquid revealed the presence of motile bacilli.

Egg 5. Similar in character to egg no. 3, but yolk a dense black throughout. White liquefied.

On 19 October six new-laid eggs were brushed on the outside with the contents of egg no. 3 whilst six others were inoculated through the egg shell, the hole made in the shell being closed with paraffin wax. These eggs were held at room temperature.

In the inoculated group one egg became patchy black in 10 days and two more in 12 days. All showed black rot in 29 days. Two of these were handed over to Dr Miles for further detailed study, together with other eggs from a

https://doi.org/10.1017/S0022172400034823 Published online by Cambridge University Press

case in which the trouble was present. None of the brushed group showed rot in 29 days, but all six were black after 44 days. There was consequently no doubt that the black rot was caused by an infective organism, and that this infection could be conveyed through the shell.

A case of imported eggs was received at Cambridge on 20 October, and candled. The position was as follows:

Sound eggs	281
Black rot eggs	46
Patchy eggs	22
Slightly patchy eggs	8
	357

The affected eggs were distributed sporadically throughout the case and not confined to the outer layers. This afforded strongly presumptive evidence that the eggs were infected prior to packing and not while in transit.

On 1 November, no further black rots had occurred in the clears retained from the case received on 20 October. One hundred and twenty-five clears were retained in the original case and the subsequent history was as follows:

	Blacks	Clears
1 Nov. 1934	5	120
10 „	15	110 (1 broken)
17 „	30	94
23 „	39	85
3 Dec. 1934	56	68
11 „	64	60
3 Jan. 1935	71	53
18 „	77	47
4 Feb. 1935	78	46

The history of this case proved clearly that eggs passed by inspection as of first-class quality could yet be infected and subsequently develope black rot.

Investigation showed that the eggs were produced under clean conditions, the feeds used being dry mash consisting of bran, pollard, lucerne meal, meat meal and limestone grit; the grain feed being cracked maize and wheat. During wet weather, however, all eggs were wiped with a damp cloth to remove blemishes, and it is obvious that, if the infective organism is one normally present in poultry excreta, etc., this method of treatment would be very effective in inoculating eggs so treated.

I. BACTERIOLOGICAL INVESTIGATION OF THE ROTTEN EGGS

Though rots of various colours in commercial hens' eggs (white, green and black) have been described as associated with the presence of bacteria, there are few detailed descriptions of the organisms responsible for any particular rot. Pennington *et al.* (1914) state that while white, spot, green and mouldy rots occur in eggs of high bacterial content, black rot, in which the egg contains very large numbers of bacteria, represents the last stages in decomposition of an egg. Turner (1927) and Levine & Anderson (1932) have described species of

Achromobacter causing mustiness in eggs, and Bohart (1930) isolated *Clostridium sporogenes* and *Cl. putrificum* from eggs with black rot. Rettger (1914) lists a large variety of bacterial species, isolated both by himself and the authors he quotes, from both fresh and stale eggs. The examination of the batch to be investigated was therefore designed to reveal a similarly large variety, including aerobic and anaerobic species and those growing at 22 and 37° C.

Method. A sample of twelve eggs, ten from the infected crate and two experimentally infected with rotten-egg contents, was examined. The eggs were opened with aseptic precautions, and about 1 c.c. of mixed white and yolk emulsified in broth. Emulsions from the first eight were immediately seeded on to three nutrient agar plates, which were incubated respectively at 22 and 37° C. aerobically, and at 37° C. anaerobically. The emulsions were then incubated for 48 hours and heated at 80° C. for 20 min. No spore-bearing organisms grew from these heated suspensions after aerobic or anaerobic incubation. All organisms appearing in the course of 6 days on the aerobic and anaerobic plates at 37° C. grew on the aerobic plate at 22° C. Some strains moreover were isolated at 22° C. that failed to grow at 37° C. Since it was clear that the eggs were not infected with obligate anaerobes, or with organisms growing only at 37° C., all subsequent primary cultures from rotten eggs were grown aerobically at 22° C. The remaining four eggs of the sample were treated in this way.

Fifty strains of bacteria were isolated from the sample and examined morphologically, culturally and biochemically. Indentities were established between strains either from different eggs, or from differently grown plate cultures of the same egg. Nine types of organism remained, exemplified by type ER 1, which is to be described later, and the following types.

ER 2. A thin, slightly curved Gram-negative bacillus, giving abundant growth of coliform colonies on nutrient agar. Ferments no sugars, but turns litmus milk alkaline in 2 days, with slight peptonization in 10 days. Indol positive, M.R. (Methyl Red test) negative, V.P. (Voges-Proskauer test) negative. Reduces nitrates, and produces H_2S. Does not liquefy gelatine.

ER 3. Thin, fusiform or coccobacillary, Gram-negative organism, giving opaque, tough or membranous, highly rugose colonies, with a cartwheel, ring, or worm-cast crenations on nutrient agar. Ferments glucose slightly; turns litmus milk alkaline. Does not liquefy gelatine.

ER 11. A non-pigmented, poorly growing Gram-positive coccus, acidifying glucose, maltose, sucrose, salicin, and litmus milk, and clotting the last.

ER 17. *Pseudomonas pyocyanea.*

ER 22*a*. A poorly growing pleomorphic Gram-negative bacillus, acidifying glucose and sucrose moderately, lactose and salicin feebly. No change in litmus milk. Gelatine; slow napiform liquefaction. Indol, M.R. and V.P., negative.

ER 29. A profusely growing, yellow pigmented Gram-negative bacillus. Acidifies glucose very feebly. Alkalinity marked in litmus milk. Gelatine not liquefied. Possibly a *Flavobacterium.*

ER 40. A profusely growing Gram-negative bacillus giving acid in glucose and maltose, and rendering litmus milk alkaline. Does not liquefy gelatine.

ER 41. As ER 40, but ferments no sugars.

Table I shows the frequency with which the types occurred in the twelve eggs. ER 1 was present in all, and its colonies always predominated in the plate cultures. ER 2, when present, was abundant. ER 3, 11, 17 and 22 were present only in moderate numbers, whilst the colonies of ER 29, 40 and 41 were scanty.

Table I. *Distribution of types of organism in twelve rotten eggs*

Type	No. of eggs in which found
ER 1	12
2	8
3	3
11	1
17	1
22*a*	2
29	1
40	1
41	1

Tests of rot-producing power

Levine & Anderson (1932) tested the effect of their organisms on fresh eggs poured into sterile flasks. Bohart (1930) used as a "test egg" segments of heat coagulated egg, about 1×1 cm., at the bottom of a tube containing a 12 cm. column of nutrient agar. Stab cultures were made, and those organisms blackening the egg fragment were deemed to be the cause of the black rot in the whole egg. This method, though it successfully removes the risk of using already infected eggs for the test, is highly artificial, besides preventing any organism but an anaerobe from growing near enough to the egg fragment to produce any effect on it. Bohart's finding that the aerobes she isolated from the rotten eggs produced no rot in the test egg is therefore to be expected. Two modifications of this method were tried; one in which a long segment of coagulated egg was embedded in agar so that the whole length of a stab culture, when made from the aerobic surface to the bottom of the tube, lay close to some portion of the egg; the other in which a segment of coagulated egg, both yolk and white, was sterilized at the bottom of a tube of broth. Neither method was successful in implicating organisms as the cause of rot, and since in any event the blackening or solution of heat-coagulated egg albumin is not comparable with the blackening of a natural egg, the artificial test egg was abandoned. Whole fresh eggs from the Experimental Farm of the Department of Agriculture, Cambridge, were therefore used. The carefully supervised conditions under which they were laid reduces the possibility of an initial contamination with a cause of black rot, but since such eggs had not been subjected to the long storing that the imported eggs had undergone, it was impossible to exclude initial infection on the grounds that it had never been observed in these eggs, and the possibility of such initial contamination was guarded against by the use of adequate controls.

(*a*) *Preliminary tests*

The eggs were washed carefully in spirit, allowed to dry, pierced over the air space, inoculated with the aid of a pasteur pipette, and sealed with sterile paraffin wax. The pipette was inserted obliquely, avoiding the yolk and leaving the inoculum close under the shell in the region of the equator of the egg. Batches of four eggs were inoculated with about 0·5 c.c. of a broth culture of each strain, two of them incubated at 22° C. and two at 37° C., except when the organism itself grew only at 22° C. when two eggs only were used. Three strains of type ER 1 and one each of ER 2, 3, 11, 17 and 18 were tested, and a control batch was inoculated with sterile broth. The eggs were stored in sterilized egg boxes with the air space uppermost and candled twice weekly.

The eggs inoculated with type ER 1 developed a black localized cloud in about 12 days, spreading towards the air space and encircling it. Fragments of opaque material then fell to the lower end of the egg and after about 30 days the egg was completely opaque, except for an enlarged air space. A hydrogen sulphide-like smell developed, reaching a maximum at about the third week. Rot developed more quickly at 22 than at 37° C. The rotten eggs yielded abundant pure cultures of type ER 1 after 4 weeks. Varying degrees, faint or moderate, of localized cloudiness developed in 4–8 weeks in the other eggs. These clouds did not spread and no egg showed a typical rot. Cultures of seven of the most cloudy of these eggs 3 months after inoculation yielded a few Gram-negative bacilli, Gram-positive cocci, and *Aspergillus fumigatus*.

In a more carefully controlled test, sixteen eggs were inoculated with two ER 1 strains and sixteen with sterile broth. Rot developed in fifteen of the infected eggs in 11 days and was marked in all by the 24th day. After 67 days two only of the control eggs showed faint clouds; cultures yielded *Asp. fumigatus*. The clear-cut nature of this result indicates that the odds against the rot being inherent in the eggs used for the test are overwhelmingly large.

(*b*) *Infectivity of type ER* 1

A 3-day 22° C. agar slope of the organism was washed off in broth. A viable count of the suspension was made in nutrient agar roll tubes and a series of tenfold dilutions inoculated, each dilution into two eggs. The results, summarized in Table II, show that very small doses of the organism can produce characteristic rot, given sufficient time. The changes in appearance on candling are correlated with the changes in appearance of the egg contents at different stages in the rot. At first the white becomes locally turbid, producing local candling opacity. The spread of liquefaction of the white corresponds with the spread of opacity. A pale brown-purple colour developes in the white at this stage, and the yolk membrane becomes thickened, appearing as a brittle white film which breaks up and fills the liquefied white as membranous flakes. These flakes consist of rounded masses of homogeneous organic matter, about 0·01–0·05 mm. in diameter, and contain very few bacteria. The yolk is stained black

Table II. *Infectivity of ER* 1

Egg	Dose	Result at 11	24	67 days
82	350,000	+ +	+ + +	C
83		+	+ + +	C
84	35,000	tr.	+ +	C
85		+ +	+ +	C
86	3,500	+ +	+ +	C
87		0	tr.	C
88	350	tr.	tr.	C
89		+ +	+ +	C
90	35	0	0	C
91		0	0	C

C = egg completely black on candling.
+ + + and + + = grades between C and +.
+ = marked localized opacity, 2–3 cm. diameter.
tr. = localized cloudiness.

at the site of the initial opacity. At this stage the candled appearance is of complete opacity. Later the whole yolk becomes less opaque, gelatinous and granular, is blackened on the outside and partially dissolved. The dissolved, blackened yolk imparts a greenish black colour to the white. This can be demonstrated by oxidation of the egg contents with hydrogen peroxide, which restores the original colour of the yolk, and reveals the white as stained yolk-yellow to a degree corresponding to the original degree of blackening. Three eggs with the specific rot examined 8 months after rotting were found filled with an acrid smelling pale green cheesy mass. In one the yolk had entirely disappeared, and in two had become a brown leathery semi-transparent flattened disc. The specific organism had died out, and the culture yielded Gram-positive cocci and feebly fermentative Gram-negative bacilli.

These experiments establish type ER 1, found in all twelve eggs, as the main cause of the rot. Type ER 2, found in eight of the twelve eggs, produced no rot. Judging by its lack of proteolytic and saccharolytic powers, it seems unlikely to take part in rot production.

It corresponds to the *Vibrio alkaligenes* of Lehmann and Neumann, described by Nyberg (1935) as commonly confused with the true *Bact. alkaligenes faecalis* of Petruschky. Its association with type ER 1 may be indicative of a common source for both, possibly faecal.

Investigation of a second sample.

One year after this first investigation, five eggs from a similar source, showing black rot by candling, were dissected and cultures made from the egg contents. The rot was typical macroscopically and ER 1 was present in all five, being the predominant organism in three and accounting for 5 and 20 per cent of the colonies developed on plates from the other two. Each plate yielded in addition lactose-fermenting coliforms and from one egg a Gram-positive coccus was isolated in large numbers.

The ER 1 strains from these five eggs were typical except that two fermented rhamnose in addition to the other sugars. Cultures of organisms very like ER 1 were also obtained from two of the eggs, differing in a ready fermentation of salicin, and in some cases a rapid fermentation of lactose. The lactose-fermenting activities of these strains were, however, very variable; lactose fermenters and non-lactose fermenters yielded both kinds of colony on subculture, and the character failed to breed true in subcultures.

THE TYPE ER 1

The results of a systematic study of the strains of ER 1 isolated from seventeen eggs are listed below. The strains form a homogeneous group, differing only in their serological behaviour. The following description is based on an examination of seventeen strains, with the exception of the flagella staining, pathogenicity and saprogenicity tests, which were made on smaller numbers.

Habitat. Isolated from rotten eggs. Has been found in road dust, farmyard manure, certain soils and human faeces.

Morphology. Straight rods $0{\cdot}5–1{\cdot}0 \times 1{\cdot}5–6{\cdot}0\mu$, with parallel sides and rounded ends, arranged singly, end-to-end pairs, and in palisades. Coccobacillary and filamentous forms are common. Staining is uniform, except in the filamentous forms, which sometimes have a coarsely vacuolated appearance. Actively motile by a single terminal flagellum $5–6\mu$ long. Gram-negative and non-acid-fast.

Agar plates. 18 hours, 37° C. Colonies are discrete, round and from 0·7 to 1·0 mm. in diameter. The surface is smooth, moist and shiny, the edge entire or very finely lobate. The structure consists of innumerable moderately fine flecks of opaque growth lying in a transparent matrix and arranged in ill-defined and broken concentric circles or rectangles; best seen by oblique illumination against a dark ground. The elevation is raised. Colour nil and opacity moderate. The colony is butyrous in consistency and easily emulsifiable.

48 hour colonies are opaque and white; in many a raised papilla appears in the centre. At 22° C. the colony grows more slowly, but to a greater final size than at 37° C.

Agar deeps. Tiny pin-point colonies develope throughout the medium, larger in a zone extending from the surface to a depth of 2–9 mm. The largest colonies appear in a definite zone about 1·5 mm. below the surface.

Gelatin stab. 24 hours, 22° C. Good growth along the stab and a fine line of liquefaction, which developes in 2 days into marked infundibuliform or saccate liquefaction, the bulk of bacterial growth settling at the bottom of the liquid. Liquefaction is complete in 3 weeks. Extensive liquefaction is accompanied by a sharp ammoniacal odour.

Broth. 24 hours, 37° C. Abundant turbid growth with a slight sediment that disintegrates entirely on shaking. In 48 hours a fine pellicle appears which

shakes easily into suspension, leaving a ring of growth on the wall of the test-tube.

MacConkey agar plates. 24 hours, 37° C. Good growth of faintly pink discrete colonies 0·7 mm. in diameter with finely lobate edge and slightly papillate centre.

Horse blood agar plates. 18 hours, 37° C. Colonies as on agar, but slightly larger; surrounded by a zone of β-haemolysis whose diameter is on the average 2·5 times that of the colony. A zone of bright red corpuscles marks the edge of the haemolytic area. On further incubation the area of haemolysis extends over the whole plate, which remains pink in colour.

Loeffler's serum. 4 days, 37° C. Growth is abundant; the slope is cracked, pitted and eroded and partly dissolved into 2 c.c. of turbid, viscid fluid.

Potato slopes. 22° C. A confluent whitish growth appears in about a week, in some strains moist and shiny, in others moist and wrinkled. In 6–8 weeks the moister parts of the growth are yellow-brown.

Resistance. 24 hour broth cultures are killed by moist heat (55° C.) in 15 min. There is no evidence of the formation of resistance spores in older cultures. Living suspensions are not dissolved by strong solutions of lysozyme.

Metabolism. Aerobe and facultative anaerobe. Growth under anaerobic conditions poor (24 hour colonies at 37° C. are flat, transparent and 0·4–0·5 mm. in diameter); both proteolysis and liquefaction of gelatin are retarded and the production of H_2S is increased in the absence of oxygen. Optimum temperature for growth 35–37° C.; on solid media the bulk of growth is finally greater at 22 than at 37° C., due apparently to earlier autolysis of the organisms in the 37° C. cultures. Filtrable haemolysin + +. A pale yellowish brown pigmentation is seen in cultures over 4 weeks old, on agar and on potato.

Nutritional. Growth is improved by the addition of blood and serum, and partly inhibited by bile salts.

Biochemical. All strains produce acid and gas in glucose, maltose, sucrose and arabinose on the first day, and in mannite in 1 or 2 days. Some strains produce acidity but no gas in the Durham's tube in lactose after 3–7 days; occasionally the indicator in these part-fermented tubes is coloured throughout the fluid, but later a reversal takes place, leaving coloured the contents of the Durham's tube only. Salicin is fermented in a few of the strains in a similar manner, but after from 10–20 days. Gas production is variable and at a maximum in 3 days. Dulcite, raffinose, xylose, inosite and rhamnose are not attacked. In 1 to 2 days litmus milk is rendered moderately acid (*p*H 6·4), the casein loosely coagulated and peptonized from above downwards. Indol and ammonia are produced from the peptone water. M.R. faintly positive (equivalent to a *p*H of about 5·6), V.P. positive. Nitrates are reduced. H_2S + in aerobic, + + + in anaerobic conditions. Catalase +, diastase +, methylene blue reductase +. Urease negative.

Serological. Seventeen strains appear on incomplete evidence (see notes below) to be related to one another, but antigenically heterogeneous.

Pathogenicity. Causes an acute septicaemic death in guinea-pigs and mice 24 hours after intraperitoneal inoculation. Filtrates of fluid cultures are toxic to mice.

Saprogenicity. Doses of over 40,000 produce characteristic rot in hens' eggs in 12 days at 22° C. Doses as small as 35 organisms eventually produce rot.

Notes on the systematic description

Habitat. Organisms similar to ER 1 have been found in various situations in England. Some were identical with ER 1, and others differed only in a readier, though slow, fermentation of lactose and salicin. Both types were agglutinated to a moderate extent by the ER 20 antiserum, and four of the strains tested produced typical rot in eggs in 14 days.

The method finally adopted for examination of specimens was as follows. The emulsion of the specimen in broth was plated on 5 per cent horse blood agar and grown at 37° C. for 24 hours. Any large, markedly β-haemolytic colony with smooth surface and entire edge, about 2 mm. in diameter, showing by brilliant, oblique illumination the typical flecked appearance, was sampled and dabbed on to the surface of a 20 per cent gelatin plate. The remainder of the colony was filmed. The gelatin plate was incubated for 2 days at 22° C., and colonies developing a 3–6 mm. pool of liquefaction were seeded into lactose, sucrose, rhamnose and litmus milk. Those cultures which within 2 days produced acid or acid and gas in sucrose, failed to ferment lactose or rhamnose, and acidified, peptonized, and clotted litmus milk, were investigated biochemically and serologically. Acidification of lactose in the Durham's tube only, in 3 or 4 days may occur with some true ER 1 strains. Feebly proteolytic, atypical organisms can be more surely excluded if a Loeffler's serum slope is included in the preliminary biochemical test; most ER 1 strains produce extensive liquefaction in 4 days.

Table III. *Occurrence of type ER* 1 *in various situations*

Specimen	Number and source of specimens	Containing ER 1
Garden soils	2, London; 1, Sussex	0
Road dust	1, London	+
Farmyard manure	1, Sussex	+
Chalk soil	1, Sussex	+
Human faeces	1, London	+
Infected meat	1, London	+
Hen faeces	20, from 11 counties	0
Hens' eggs, "bad"	1, London; 3, Cambridge	+, 0

Table III summarizes the result of these investigations. The occurrence of ER 1 in road dust, farmyard manure and human faeces, suggests a faecal habitat for the organism, and the occurrence of moderate amounts of agglutinin for an ER 1 strain in English hen sera (see below) suggests that English hens may be carriers of this organism. Accordingly twenty specimens of freshly gathered hen faeces, each specimen from a different flock, were collected from

the following localities. Bucks, 1; Cambs, 1; Cheshire, 2; Essex, 2; Hants, 2; Herts, 2; Kent, 2; Lincs, 2; Norfolk 4; Notts, 1; and Suffolk 1. On blood agar the predominant colony from almost all specimens was that of a small α-haemolytic, highly pleomorphic, *Streptococcus*. Coliforms, white staphylococci, and gelatin-liquefying Gram-negative bacilli were common. Gram-positive spore bearers occurred in a few specimens. Special attention was paid to two specimens from hens known to frequent a general farmyard where they came into contact with plenty of mammalian excreta. In these, and in all the others no ER 1 was found.[1]

The human faecal strain occurred as the only abnormal organism in the faeces from a case of chronic colitis. No causal relationship between the disease and the organism was demonstrable. The infected meat strain was the predominant aerobic organism found in a meat pie responsible for a death from botulism (Aitken, *et al.*, 1936). These last two strains and two of the soil strains produced typical black rot in eggs.

Colony form. Two variants have been noted; one, which appears more frequently in older cultures, is large with a moist granular surface and a finely lobate edge; the flakes of opaque material are large and angular; individual organisms are more filamentous than usual. This "rough" variant grows in broth as a thick sediment made up of tangled filamentous forms. The second variant is smaller than normal and has a smooth surface and is cheesy in consistency; the individual organisms are thick and dark staining.

Resistance to lysozyme. In view of the possible role that the bactericidal action of egg-white, recorded by Rettger & Sperry (1912), might play in the immunity of the egg to infection, type ER 1 was tested for susceptibility to lysozyme, the bacteriolytic principle in egg-white described by Fleming (1922). Living suspensions, about 5×10^9 per c.c., of ten strains were not lysed by a 1/10 dilution of fresh egg-white that contained enough lysozyme to dissolve a similar suspension of the susceptible *Micrococcus lysodeikticus* when diluted to 1/100,000. The reaction was carried out at 37° C.

Haemolysin. A 1 : 5 dilution of a Seitz E.K. filtrate from a 24 hour broth culture lysed an equal volume of 5 per cent horse blood in 1 hour at 37° C. The haemolytic titre of the filtrate was not improved on reduction by sodium sulphite (1 mg. to the c.c., 1 hour at reduced pressure).

H_2S *production*, under aerobic and anaerobic conditions was estimated by the amount of blackening of lead acetate papers fixed at a constant distance above the surface of the broth cultures.

Diastase production. Strains grown on nutrient agar plates containing 2 per cent starch, incubated 2 days at 37° C. and tested with iodine solution. 2 mm. colonies produced a 10 mm. area of starch destruction.

[1] During the course of the investigation, four "bad" English hens' eggs were examined. One exhibited typical black rot, and yielded on culture type ER 1 predominating, and small numbers of *Ps. pyocyanea* and an atypical coliform. The egg was bought from an itinerant dealer professing to deliver new-laid English eggs; its English origin could not be established.

Urease production. Tested by growth in 1 per cent peptone water containing per cent urea; and by adding a thick washed suspension of organism to a per cent solution of urea in saline. No breakdown occurred in the former est. After 96 hours at 37° C. the thick suspension (about 10^9/c.c.) produced mmonia equivalent to 0·07 per cent of the urea present (Nessler's colour stimation used). *Proteus vulgaris* under similar conditions broke down 6 per cent of the urea.

Pathogenicity. Guinea-pigs. Two received 0·2 c.c. of a 24 hour broth culture nd died in 18 hours. Organisms were present in large numbers in the heart lood. The strain ER 20 was used.

Mice. A viable count was made of a suspension of a 3 day agar culture and enfold dilutions injected intraperitoneally each into batches of four mice.

Table IV. *Pathogenicity of ER* 20 *for mice*

No. of agar-viable organisms	Deaths	Last death in (days)
35×10^6	4/4	1
35×10^5	4/4	2
35×10^4	0/4	—
35×10^3	1/4	2
35×10^2	1/4	4

The organism was recovered from the heart blood of all the dead mice.

Toxicity. A Seitz filtrate of a 4 days' cooked meat medium culture kills nice in 2 days after intraperitoneal injection of 0·05 c.c.

Serology. A limited investigation only was made. The following observations re of interest. Two sera were prepared against one strain; a formolized suspension produced a serum of a slightly higher titre than a phenolized suspension. The agglutination at 56° C. is muddy, midway in type between Salmonella H and O agglutination; the end-point is maximum in 24 hours. Table V (*a*) shows the titres of each of the organisms with the serum prepared against ER 20 (formolized). After some months the serum was retested, and the titres against ER 1, 6, 35 and 42 found to have dropped 128-, 128-, 34- and 24-fold respectively. The other titres had dropped about fourfold, and the agglutination was muddy except with ER 20, which gave well-defined deposits in a cleared fluid. The later five strains agglutinated as recorded in Table V (*b*). There is no correlation between the degree of agglutinability and the minor differences in late fermentation of salicin and lactose.

Table V. *Agglutination titres of the ER* 1 *strains with an antiserum against strain ER* 20

(*a*)				(*b*)	
Strain	Titre	Strain	Titre	Strain	Titre
ER 1	640	ER 27	50	ER 71	160
6	640	35	640	72	640
10	1600	42	480	74	20
13	80	44	20	76	40
15	20	47	100	77	?10
20	3000	49	20		

An attempt was made to see how much of the agglutination was due to the flagellar antigens and how much to somatic. On the assumption that the alcohol treatment which destroys flagellar antigens in the Salmonella group would be as efficacious with this organism, agar cultures of four strains were washed off with alcohol, heated for 1 hour at 56° C., suspended in saline and their agglutinability compared with that of untreated suspensions. There were from four- to eightfold reductions in titre (for example, from 1 : 640 to 1 : 80, 1 : 480 to 1 : 120). It appears that a greater part of the agglutinin response is due to flagellar antigens. This point was not further investigated.

The position of type ER 1 in the Schizomycetes

The organism is rod-shaped and motile by a polar flagellum. Gram-negative. Non-sporing. Grows well at 37° C. on ordinary media without the production of a definite pigment. Aerobe and facultative anaerobe. Proteolytic and liquefies gelatin. Actively ferments carbohydrates with the production of acid and gas.

It corresponds to no organism described by Bergey (1934). If the not very characteristic yellowish coloration of old cultures is disregarded and the organism considered as a non-pigment producer, for the purposes of classification two groups remain for consideration,[1] *Achromobacter* and *Proteus*. The Erwinieae, consisting of plant pathogens, contains species in general like type ER 1, and there is no valid reason why an organism belonging to this tribe should not cause egg rot. Indeed, in the investigation of any abnormal flora in a given situation, the reservoir from which the organism is derived may be a habitat entirely foreign to that in which it is found. But the listing of *habitat* as a prominent character of group automatically determines the exclusion of an organism from a group so defined, unless it is obviously related to, or identical with members of that group, or is found in that habitat. For this reason the Erwinieae were not further considered.

The Achromobacterieae include soil organisms, and in general grow best at 20–25° C. ER 1 has no marked resemblance to the type species, though it differs from no. 54 in Bergey's Genus VII, *A. sulfureum* (an organism of undetermined flagellation), in producing indol and growing best at 37° C.

There is very little direct resemblance to the type species of the *Proteus* group, *P. vulgaris*. ER 1 has one flagellum; does not swarm on moist agar; ferments mannite and occasionally ferments lactose to a slight degree; and does not break down urea. (*P. vulgaris* itself can produce a black rot. Four typical strains from human source were tested and two of them produced in eggs a typical foul-smelling black rot, which did not, however, develop until 12 weeks after inoculation.)

Its *Proteus*-like, as against its *Achromobacter*-like characteristics are as follows: It grows best at 37° C.; it grows well on MacConkey's agar; it produces

[1] The feeble yellow pigment may indicate the genus *Flavobacterium*, but the organism fails to fit the group in other respects.

acid and gas vigorously from carbohydrates. It ferments maltose and produces indol and H_2S; is haemolytic and proteolytic; and is capable of multiplication in warm-blooded animals, and of killing them.

It may be mentioned here that an attempt was made to produce variants of ER 1 with peritrichous flagella, both by allowing strains to grow through tubes of 0·3 per cent agar (Colquhoun & Kirkpatrick, 1932) and by twice daily subculture in broth for a period of 3 weeks. No increase in the number of flagella occurred in the resulting strains. However, the *Proteus* group already contains bacteria with a single polar flagellum, namely *P. noctuarum* and *P. sphingidis* (Bergey, 1934; Genus XIX, nos. 4 and 9). Both differ in producing alkali in litmus milk, failing to reduce nitrates, producing no indol or acetyl-methyl-carbinol, and fermenting xylose (White, 1923). ER 1 more closely resembles *P. hydrophilus* and *P. bombycis* (Bergey, Genus XIX, nos. 5 and 7). The former possesses peritrichous flagella. The flagellation of the latter is unrecorded, but it produces no diastase and no haemolysin, and indol is not formed from peptone water cultures (Glaser, 1924). No organism similar to the type ER 1 has been recorded in the published papers that have been searched. ER 1 appears to be a hitherto undescribed species, and in view of its probable economic importance, a name is desirable. It is proposed to place it in the genus *Proteus*, with the specific name *melanovogenes*, indicating its power of producing blackening of eggs.

II. THE MODE OF INFECTION OF THE EGGS

The possible modes of infection can be conveniently divided into two classes; the extra-genital and genital. Neither of these hypotheses can be verified in the laboratory, though the mechanisms can be proved valid for given artificial conditions. There is experimental evidence that bacteria can penetrate the apparently normal unbroken shell of a hen's egg (Lange, 1907; Rettger, 1914; Wilm, 1895; Zörkendörfer, 1893). The power of penetration depends on the time of exposure to the culture, the size and motility of the organism, and the presence of a layer of a "gelatinous" substance on the shell of fresh eggs. Motile organisms penetrate more easily than non-motile; the eggs were soaked for periods varying from 15 hours to several days. The gelatinous layer is spoiled by damp and readily removed by soap and water washing. Damp conditions are associated with a high incidence of bacterial rots (Rettger, 1914), and it is noteworthy that Pennington *et al.* (1914) found a higher bacterial content in eggs with stained shells, indicating wetting at some period, than in those with the shell merely soiled, however heavily, by hen faeces. The commercial hen's egg is laid in an environment rich in bacteria, a number of which, given a means of entry, can readily infect the egg.

The genital infection is possible. Horowitz (1913), concluded that hen oviducts were sterile, but recent work on the occurrence of the Salmonellas in ducks' eggs shows that infected eggs were laid by birds which, though ap-

parently healthy were in fact heavily infected with *Bact. aertrycke* or *enteritidis* (Scott, 1933; Bruns & Fromme, 1934; Fromme, 1934). The bacteria were isolated from the gut and a number of organs, including the ovaries; and their presence in large numbers in both the yellow and the white of the eggs soon after laying favours an internal infection of the egg rather than an extra-genital penetration of the shell by excreted bacteria.

The demonstration of *Proteus melanovogenes* in the rubbish of a hen-run would favour neither hypothesis, since the organism could equally well be excreted by infected hens or derived from a different source.

Experimental

(*a*) *Penetration of the egg shell by* P. melanovogenes

The experiment of Mr E. T. Halnan reported above shows that rotten egg contents produced rot when painted on to fresh eggs. The infectivity of 24 hour broth cultures of strain ER 20 was investigated. Fresh eggs from the experimental farm of the Department of Agriculture, Cambridge, were dipped for varying times into the broth. They were allowed to drain and dry in air and held at 22° C. in the dark. Table VI summarizes the treatment of several batches of eggs. All the rotten eggs were cultured and found predominantly infected with *P. melanovogenes*. Painting with rotten white and yolk of an infected egg is more nearly analogous to the soiling of eggs by bacteria-containing mud and faeces, but seven fresh untreated eggs so painted failed to develope rot within 50 days.

Table VI. *Experiments on infection of egg through shell*

Treatment	Duration of dip in culture	No. of eggs used	Number becoming rotten
Dipped in sterile broth	Nil	9	0
Nil	5 sec.	9	0
Nil	5 min.	4	1 (12 weeks)
Scrubbed with soap and water; rinsed in spirit	1 min.	4	4 (8 weeks)
Scrubbed with soap and water; rinsed in spirit	25 min.	4	2 (12 weeks)
Scrubbed with soap and water; soaked in 1/500 HgCl 20 min.; washed, rinsed in spirit	5 min.	4	3 (12 weeks)

Eggs observed for 6 months.

The experiments show that the organism can penetrate the shell in a few minutes in wet conditions.

(*b*) *Attempted experimental bacterial infection*

Four 1-year-old white leghorn laying hens were kept in bin cages, 20 in. in diameter, 16 in. high, containing a 4 in. layer of wood chips. They were fed on a diet consisting of middlings 2½, bran 1, Sussex ground oats 1, maize meal 1 (parts by weight) to which were added Soya bean meal 4 per cent, fish meal

4 per cent, and mineral mixture 3 per cent. Water was changed daily and the cages cleaned and sterilized three times weekly.

Hens I and II were fed with the equivalent of $1\frac{1}{2}$ 24 hour agar slopes of strain ER 20, mixed into the meal on the 1st and 4th day of the experiment. Hens III and IV received respectively about 100×10^6 and 20×10^6 viable organisms intraperitoneally on the 1st day. Eggs were collected with sterile precautions, washed in spirit, dried and stored in the dark at room temperature. Six eggs chosen at random from under similar hens living in open conditions on the experimental farm were soap-washed and stored as a control at the

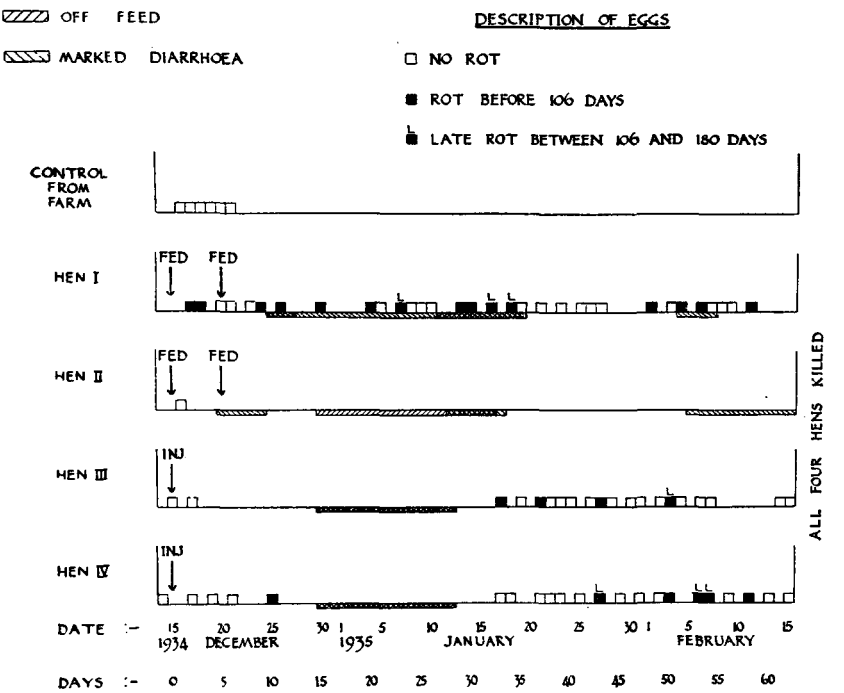

Fig. 1. Attempted production of specific black rot in eggs of four hens. Strain ER 20.

beginning of the experiment. The hens were observed for 63 days, and the results of the experiment are summarized in Fig. 1. Of the bacteria-fed hens no. II ceased to lay and at times had diarrhoea and was off its feed. No. I, though similarly affected, laid 32 eggs both in the healthy and the sick periods. The fluid excreta of these hens were twice examined during the first bouts of diarrhoea, and yielded only lactose-fermenting and non lactose-fermenting, acid and gas-producing coliform organisms; *P. melanovogenes* was not isolated. The injected hens both fell sick with diarrhoea, fed badly and ceased laying. They recovered and started laying again on the 33rd day, and continued in apparent good health to lay to the end of the experiment. During the bouts of diarrhoea, all the hens were badly soiled ventrally and on the breast feathers,

and the cages, in spite of frequent cleaning, became very dirty. All four hens were killed on the 63rd day. Circumstances prevented the full examination of the birds, but no gross abnormalities were seen post-mortem, either in the ovaries or elsewhere. The blood and peritoneal fluids were bacteriologically sterile.

There is thus no direct evidence that the hens were infected with *P. melanovogenes*. The illness might have been due to the change from open to cramped and dirty conditions. But the serum, taken at death, contained agglutinins against ER 20, showing that there had been a response to the antigens in the organism. The titres were as follows:

Hen I	...	...	...	320
Hen II	...	...	...	240
Hen III	...	...	...	320
Hen IV	...	...	...	160

A standard for comparison with untreated hens was obtained by titrating the sera of 100 hens, representing five separate English flocks, against the same suspension. The sera were chosen from larger batches at random, except that those known to contain agglutinins against *Bact. pullorum* were rejected. Table VII shows a distribution of end-points in the titration of these sera. 4 per cent have a titre against ER 20 of 1/160 or more, so that, assuming these 100 sera to be a representative sample from normal hens, the probability, p, of such a titre turning up in any one hen is 0·04. The four experimental hens all yielded sera with titres of 1/160 or more. The probability of such a finding occurring by chance is p^4, which corresponds approximately to odds of 1 in 400,000.

Table VII. *Distribution of titres against ER* 20 *in* 100 *normal hen sera*

	Titre of serum						
	Less than 5	5	20	40	80	160	320
No. of sera	24	39	23	3	7	3	1

The experimental titres therefore appear to signify at any rate an abnormality of the hen sera, due to a response to the antigens of the strain used in the experiment. The injected hens, judging from the ease with which a rabbit responds to dead antigen, might have achieved this titre as a result of the original dose, rather than of an infection, but the bacteria-fed hens may have been invaded and infected by the organism to a certain extent.

The results of the examination of the eggs are summarized in Table VIII.

Table VIII. *Eggs laid by farm hens infected with ER* 20

		Eggs		
Hen	Infected	Total laid	Total going rotten	% going rotten
I	By mouth	32	15	46·8
II	By mouth	1	0	—
III	Intraperitoneally	21	4	11·4
IV	Intraperitoneally	24	6	25·0

https://doi.org/10.1017/S0022172400034823 Published online by Cambridge University Press

The eggs were candled periodically. Atypical darkening appeared in some after 70 days, and the darkened eggs were examined bacteriologically 106 and 180 days after the beginning of the experiment. None contained *P. melanovogenes*.

The rots were mainly of two kinds; a blackened or partially blackened yolk with a brownish purple white, or a yellow-brown yolk and white. A few eggs, normal by candling, were examined and about half had green whites. There was no correlation between the type of rot and the bacteriological contents, except that the green whites yielded *Ps. pyocyanea*, nor were any of the organisms isolated found in particular association with one another. Of the twenty-five rotten eggs eleven yielded a pure culture or a mixture of similar types of organisms. The variety of the organisms isolated is seen in Table IX. Full identification was made only with the *Ps. pyocyanea*.

Table IX. *Organisms isolated from twenty-five eggs of infected hens*

Type of organism	Gram stain	No. of eggs in which found
Coliforms: Acid and gas from carbohydrates; L.F. and N.L.F.	Negative	12
Bacilli: Acid only from carbohydrates; gelatin-liquefaction positive or negative	Negative	6
Ps. pyocyanea	Negative	9
Bacilli: Acid in glucose only, or non-fermenters of the *Bact. alkaligenes* type	Negative	8
Cocci	Positive	5
Bacilli: Non-sporing	Positive	2

Three conclusions can be tentatively drawn from this experiment. Firstly, the higher incidence of rots in eggs laid during the bouts of diarrhoea demonstrates experimentally that non-specific rot is more likely to occur in eggs laid in dirty conditions than eggs laid in clean. Secondly, the conception of black rot as the last stage of decay of an egg grossly infected with bacteria is invalid under the conditions of the experiment, in which eggs grossly infected with a large variety of bacteria failed, during the course of several months, to develope the candling or putrefactive changes associated with true black rot. It is probable that black rots are all due to organisms having biochemical properties like those of *P. melanovogenes*. Thirdly, there is evidence to suggest that hens can be infected with *P. melanovogenes*. Its agglutination by relatively high dilutions of a proportion of normal English hen sera suggests that infection might be present even in hen communities in whose eggs this type of black rot is uncommon.

The experiment does not exclude the possibility of a genital infection of eggs by *P. melanovogenes*, since the number of hens used was small and the hens were incompletely investigated. A much more extensive test would be required to disprove this hypothesis.

III. COMMENT

Although it has been established that *P. melanovogenes* can infect eggs, its source, general distribution and the conditions necessary for infection remain undetermined. The organism is markedly putrefactive and has a number of the qualities that characterize pathogens for warm-blooded animals, and on these grounds may conceivably be naturally pathogenic for hens, producing a mild enzootic disease, and causing the hens to lay infected eggs. In this connexion, the most significant finding is the relatively high titres in English hen sera against the strain ER 20. This strain is, however, one of an antigenically heterogeneous group, of which other members might yield different titre distributions against hen sera. The point was tested in the following experiment. Thirty *pullorum*-negative hen sera from various flocks were tested in dilutions of 1/20 and 1/40 against four suspensions, namely, ER 20 and three other *P. melanovogenes* strains which were poorly agglutinated by an ER 20 serum. Total agglutination occurred more frequently with ER 13 and 20, whilst ER 27 and 47 at best formed a muddy deposit in a partially agglutinated suspension. Taking any degree of agglutination in either dilution as positive, twenty-four of the thirty sera were positive; twenty-two against ER 20,

Table X. *Association of agglutinins against four strains of type ER* 1 *in thirty hen sera*

Agglutination of strain	Associated with agglutination of strain			
	ER 13	ER 20	ER 27	ER 47
ER 13	14	12	7	3
ER 20	—	22	7	3
ER 27	—	—	9	3
ER 47	—	—	—	3

fourteen against ER 13, nine against ER 27 and three against ER 47. Table X shows the association of positive agglutination of one strain with that of the others. ER 47 was agglutinated only when the rest were; ER 27 when ER 13 or ER 20 were; and ER 13 when ER 20 was, with two exceptions. This very rough test indicates that, whatever the strains that have produced these titres in English hens, their antigens are well represented by those of ER 20, and no important change in the titre distribution is obtained by the survey made with other strains. Before, however, representative organisms could be selected to investigate this problem fully, a complete serological survey of the known rot-producing strains would be necessary, employing the technique of agglutinin adsorption.

Alternatively, the organism may be derived from the environment of the hen, and not directly associated with the hen itself; it has been found in places remote from hens and may have a widespread habitat. Infection of the egg would then depend not on its mere presence in the egg's immediate surroundings, for in that case the majority of eggs would become infected, but on special conditions, such as the wiping of soiled eggs with an infected damp cloth, as suggested by Mr E. T. Halnan in the introduction.

IV. SUMMARY

1. An organism is described as a specific cause of *black rot* in two consignments of hens' eggs imported into England.

2. It produces the rot experimentally when inoculated into fresh eggs, and penetrates apparently normal egg shells.

3. Four hens inoculated with the organisms laid eggs that failed to develop the specific rot. The number of hens used was small, and no definite conclusion about the mode of infection can be drawn from the result of this experiment.

4. The organism is found in English soils and manures.

5. Eleven of 100 English hen sera agglutinated the organism in dilutions of 1 in 80 to 1 in 320.

6. The organism is provisionally placed in the *Proteus* group, and the name *Proteus melanovogenes* is assigned to it.

ACKNOWLEDGEMENTS. I am greatly indebted to Mr E. T. Halnan of the Department of Agriculture, Cambridge, for the supply of hens and hens' eggs used in the experiments described in this paper; to Dr C. A. McGaughey of the Veterinary Research Laboratories, for organizing the collection of hen faeces and for samples of hen sera; to Mr A. P. Sinker for the specific name given to the organism; to Dr T. L. Swenson of the U.S. Department of Agriculture, Washington, for the gift of certain Bulletins; and to Prof. H. R. Dean for his interest and encouragement.

REFERENCES

AITKEN, R. S., BARLING, B. & MILES, A. A. (1936). *Lancet*, ii, 780.
BERGEY, D. H. (1934). *Manual of Determinative Bacteriology*. 4th Ed.
BOHART, R. M. (1930). *Amer. J. Hyg.* **11**, 168.
BRUNS, H. & FROMME (1934). *Münch. med. Woch.* **81**, 1350.
COLQUHOUN, D. B. & KIRKPATRICK, J. (1932). *J. Path. and Bact.* **35**, 367.
FLEMING, A. (1922). *Proc. Roy. Soc.*, Ser. B, **93**, 315.
FROMME (1934). *Deut. med. Woch.* **60**, 1969.
GLASER, R. W. (1924). *J. Bact.* **9**, 339.
HOROWITZ (1913). Quoted by Rettger (1914).
LANGE, R. (1907). *Arch. f. Hyg.* **62**, 201.
LEVINE, M. & ANDERSON, D. Q. (1932). *J. Bact.* **23**, 337.
NYBERG, C. (1935). *Ztrbl. f. Bakt.* Abt. I, Orig. **133**, 443.
PENNINGTON, M. E., JENKINS, M. K., ST JOHN, E. Q. & HICKS, W. B. (1914). *Bull. U.S. Dept. Agric.* No. 51.
RETTGER, L. F. (1914). *Ztrbl. f. Bakt.* Abt. II, **39**, 611.
RETTGER, L. F. & SPERRY, J. A. (1912). *J. Med. Research*, **28**, 315.
SCOTT, W. M. (1933). Reported in *Off. International d'Hyg. Publique*, **25**, 828.
TURNER, A. W. (1927). *Australian J. Exp. Biol.* **4**, 57.
WHITE, G. F. (1923). *J. Agric. Research*, **26**, 487.
WILM (1895). Quoted by Rettger (1914).
ZÖRKENDÖRFER (1893). Quoted by Rettger (1914).

(*MS. received for publication* 9. x. 1936.—Ed.)